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(54) Title: EXPRESSION AND PURIFICATION OF RECOMBINANT SOLUBLE TISSUE FACTOR

(57) Abstract

A method is disclosed to make any protein in a form that can be isolated rapidly from a solution using a specific monoclonal antibody designated "HPC-4". It has now been determined that it is possible to form a fusion protein of the epitope with a protein to be isolated, and isolate the protein using HPC-4-based affinity chromatography. In the preferred embodiment, a specific protease cleavage site is inserted between the epitope and the protein so that the epitope can be easily removed from the isolated protein. In an example, a functionally active soluble tissue factor including the twelve amino acid epitope recognized in combination with calcium by HPC-4 and a factor Xa cleavage site was expressed from a vector inserted into a procaryotic expression system. The recombinant tissue factor can be rapidly isolated in a single chromatographic step using the HPC-4 monoclonal antibody immobilized on a suitable substrate. Once isolated, the Protein C epitope is removed by cleavage with factor Xa, leaving the functionally active, soluble tissue factor.

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EXPRESSION AND PURIFICATION OF RECOMBINANT SOLUBLE TISSUE FACTOR

The United States government has rights in this invention by virtue of grants from the National Institutes of Health, grant numbers R01 HL44225 and R01 HL29807.

This invention is generally in the area of methods for purifying proteins, especially blood clotting proteins, using recombinant technology and an unique epitope of a monoclonal antibody directed against Protein C zymogen.

Methods for purifying proteins have been used for many years and can be generally divided into chromatographic methods, for example, ion exchange chromatography, molecular weight sieving, high pressure liquid chromatography, affinity chromatography, and electrophoretic methods, for example, electrophoresis on agarose or acrylamide gels and isoelectric focusing. The usual disadvantages of all of these methods are that they require the starting material be passed through several processes to remove contaminants to the point where the desired material is substantially pure.

In immunoaffinity chromatography, an antibody to the desired protein or other molecule is immobilized on a chromatographic substrate, the protein mixture is applied to the substrate under conditions allowing the antibody to bind the protein, the unbound material is removed by washing, and the bound protein is eluted using, for example, high or low pH, protein denaturants or chaotropes. The end result is a substantially pure protein which often lacks full biological activity.

A variation of this method is described in U.S. Serial No. 07/730,040 filed July 12, 1991, which is a continuation of U.S. Serial No. 07/292,447 entitled "Monoclonal Antibody against Protein C" filed December 30, 1988 by Charles T. Esmon and Naomi L. Esmon,

disclosing the properties of the monoclonal antibody, HPC-4. The hybridoma cell line which s cretes the monoclonal antibody designated as HPC-4, was deposited with the American Type Culture Collection, Rockville, MD, on November 2, 1988, and assigned ATCC No. HB 9892. This deposit is available to the public upon the grant of a patent.

HPC-4 binds protein C, not activated protein C (APC), and only in the presence of calcium. Thus, when the antibody is immobilized on an affinity support, protein C can be isolated from either plasma-derived sources or from tissue culture expression systems under extremely mild conditions. This is important in maintaining the biological activity of the product and the stability of the solid support resin. Because activated protein C is not bound under any conditions, the resulting product is completely free of APC.

The antibody binds to a defined region of the protein C molecule that is contained within residues 6 and 17 of the heavy chain, specifically E D Q V D P R L I D G K. This peptide can be immobilized directly on a solid support resin and can be used to isolate the antibody in high concentrations from mouse ascites fluid or tissue culture supernatants. This approach allows the isolation of the antibody in extremely pure form in high yield, even from very dilute solutions. The antibody can be removed from the solid support peptide either by the removal of calcium ions, if desired, or by 1.5 M guanidine, which does not affect the function of the purified monoclonal antibody.

It would be advantageous if the purification methods using the Protein C epitope in combination with HPC-4 could be applied to the purification of other proteins, especially blood clotting proteins. One such system using a completely different antibody has been described by Prickett, et al., BioTechniques

7(6), 580-589 (1989), using a calcium-dependent antibody that recognizes several of the enterokinase sites that are used for Ca^{2+} coordination, including residues 1,2,3,4, and 7 from Fig 4, p. 583 and Table I, p. 586. This sequence is attached to the N-terminus of the protein to be isolated, and subsequently removed by treatment with urokinase.

It is therefore an object of the present invention to provide a method and means allowing isolation of purified proteins, especially blood clotting proteins, in a single chromatographic step.

It is a further object to provide recombinant proteins having an amino acid sequence specifically bound by a monoclonal antibody.

Summary of the Invention

A method is disclosed to make any protein in a form that can be isolated rapidly from a solution using a specific monoclonal antibody designated "HPC-4". HPC-4 binds a twelve amino acid epitope of Protein C zymogen recognized in combination with calcium. It has now been determined that it is possible to form a fusion protein of the epitope with a protein to be isolated, and isolate the protein using HPC-4-based affinity chromatography. In the preferred embodiment, a specific protease cleavage site is inserted between the epitope and the protein so that the epitope can be easily removed from the isolated protein. In the most preferred embodiment, the fusion protein is formed by expression of a recombinant gene inserted into an appropriate vector.

In an example, a functionally active soluble tissue factor including the twelve amino acid epitope of Protein C zymogen recognized in combination with calcium by a specific antibody to Protein C zymogen ("HPC-4") and a factor Xa cleavage site was express d from a vector ins rted into a suitable pr caryotic or

eukaryotic expression system. The recombinant soluble tissue factor can be rapidly isolated in a single chromatographic step using the HPC-4 monoclonal antibody immobilized on a suitable substrate. Once isolated, the Protein C epitope is removed by cleavage with factor Xa, leaving the functionally active, soluble tissue factor.

Brief Description of the Drawings

Figure 1 is expression vector pIN-III-*pelB* encoding the HPC-4 epitope and factor Xa cleavage site, followed by several restriction enzyme sites for insertion of the gene encoding the protein to be isolated.

Figure 2 is a schematic representation of steps involved in the construction of pIN-III-*pelB*-tTF expression vector. Subcloning of tTF was performed in two stages. At the first stage an intermediary plasmid was prepared which harbored the tTF gene (pUC-tTF). At the second stage the tTF gene was removed from pUC-tTF and subcloned into the pIN-III-*pelB* (see the text for more detail). The resulting pIN-III-*pelB*-tTF was used for the transformation of *E. coli* (XL1-B) for expression.

Figure 3 is a photograph of an SDS gel electrophoresis of the tissue factor, which runs as a monomer in the gel even without disulfide bond reduction.

Figure 4 is a graph of Xa generation rate (m absorbance/min/min) versus tTF (nM) for TTF-HPC-4 (open circles); TTF-293 (closed circles); and TTF-Xa-DIG (open squares).

Detailed Description of the Invention

A fusion protein readily isolated by affinity chromatography using HPC-4 antibody is prepared by insertion of a DNA sequence encoding the twelve amino

acid HPC-4 epitope into a vector, followed by the gene encoding the protein to be isolated. In the preferred embodiment, a specific protease cleavage site is inserted into the vector between the epitope and protein coding sequence, so that the resulting fusion protein can be easily cleaved to yield the epitope peptide and the desired protein.

In the following non-limiting example, a nucleic acid sequence encoding the twelve amino acid residue epitope for HPC-4 is inserted into an expression vector in an orientation such that the expression protein contains the HPC-4 epitope followed by a factor Xa cleavage site and then the amino terminus of tissue factor which has had the signal peptide, cytosolic tail, and transmembrane spanning domains deleted.

The HPC-4 Monoclonal Antibody

The HPC-4 antibody and uses thereof are described in U.S. Serial No. 07/730,040 filed July 12, 1991, which is a continuation of U.S. Serial No. 07/292,447 entitled "Monoclonal Antibody against Protein C" filed December 30, 1988 by Charles T. Esmon and Naomi L. Esmon, the teachings of which are incorporated herein. A detailed analysis of the properties of the HPC-4 monoclonal is presented in Stearns, et al., "The Interaction of a Ca^{2+}-Dependent Monoclonal Antibody with the Protein C Activation Peptide Region," J. Biol. Chem. 263, 826-832 (1988).

The HPC-4 monoclonal antibody is directed against a peptide sequence present in the activation region of the heavy chain of Protein C and Ca^{2+}. This peptide sequence consists of twelve amino acids, glutamic acid-aspartic acid-glutamine-valine-aspartic acid-proline-arginine-leucine-isoleucine-aspartic acid-glycine-lysine (E D Q V D P R L I D G K) (Sequence ID No. 1). An advantage of this sequence is that it is short enough to be made synthetically but

long enough to impart specificity, thereby avoiding potential cross reactions between the antibody and proteins other than the fusion protein.

The antibody appears to have at least one metal ion binding site in addition to the peptide binding site. The peptide binding activity is responsive to, or "dependent on", binding at the metal ion binding site. The metal ion binding site is capable of binding to a divalent metal cation such as calcium, or a metal having a similar ionic radius and coordination properties such as Tb^{3+}. The peptide does not bind Ca^{2+} and hence no Ca^{2+} binding site is added to the fusion protein. This will minimize nonspecific Ca^{2+} mediated interaction with the isolation matrix potentially inherent in the Prickett approach, described at page 3. In the case of tissue factor, it also means that calcium binding to factor VII (the ligand for tissue factor) can be studied without interference due to an additional metal binding site in the tissue factor fusion protein.

When calcium binds to the metal ion binding site in the antibody, the monoclonal antibody becomes significantly more receptive to binding to the peptide. When a metal ion is not bound to the metal ion binding site of the monoclonal antibody, the antigen binding site is relatively unreceptive to binding the antigen. Accordingly, antibody-antigen binding may be controlled by varying the metal ion concentration in the media surrounding the antibody.

Proteins to be Expressed and Purified.

The method described herein is not limited as to the protein that can be expressed as a fusion protein, isolated using HPC-4, then separated from the epitope as the pure protein.

Vectors and Expression Systems

A vector is selected for expression of a sequence encoding the pelB leader peptide HPC-4

epitope and protein to be isolated, preferably separated by a specific protease cleavage site. Examples of suitable bacterial expression vectors which are commercially available include pcDNA II (Invitrogen #V400-20), pNH8a (Stratagene, #215201) and pBTac1 (Boehringer Mannheim, #1081365). These vectors are used for expression of full length or partial cDNA sequences in bacteria such as *E. coli*, where the expressed protein usually accumulates as insoluble aggregates in the cytoplasm of bacteria called inclusion bodies. To extract the target protein, a high concentration (usually 8 M) of a chaotropic agent such as urea is required to dissolve inclusion bodies. This process denatures the protein and results in the inactivation of the target protein. To obtain a functional protein, a refolding step is necessary which is usually very inefficient and difficult to control. Examples of commercially available mammalian expression vectors include pRc/RSV (Invitrogen, #V780-20), pRc/CMV (Invitrogen #V750-20), and pMC1NeO (Stratagene #213201). These expression vectors usually contain a suitable promotor that can direct high-level expression of recombinant proteins in mammalian cells and they also contain a drug resistant gene that can be used for selection of those mammalian cells that have integrated these vectors into their genomes. These vectors are suitable for the expression of full length cDNA and any other DNA fragment which contains a leader peptide at the 5′ end of the sequence. These constructs are transferred into a suitable expression system, either procaryotic cells such as *E. coli*, or eukaryotic cell, such as a yeast or mammalian cell culture system.

It is also possible to insert the cDNA encoding th fusion protein into an embryo for production of a transgenic animal for production of the protein using known methodology. The protein expression can be

targeted to a specific tissue using a tissue specific promotor in combination with the protein encoding sequence. For example, the fusion gene is isolated on 1% agarose gel followed by electroelution in a dialysis bag, as described by Maniatis, et al. (1982). The eluted DNA is precipitated, redissolved in water and purified by passing through an elutip-D column as per the instructions of the manufacturer (Schleicher and Schuell, Inc., Keene, NH). The purified DNA is dissolved in 5 mM Tris (pH 7.4) and 0.1 mM EDTA at 3 μg/ml concentration for microinjection.

Mice or other suitable animals such as rabbits or sheep embryos are obtained from commercial suppliers. Reagents such as bovine serum albumin, gelatin, and pronase are obtained from Sigma Chemical Co., St. Louis, MO. Hormones for superovulation, PMS and hCG, are obtained from Organon, Inc., NJ. Hyaluronidase is purchased from Sigma. Restriction enzymes are obtained from New England Biolabs, Beverly, MA. The micromanipulator made by Nara Shige, USA, Inc., Rainin Instruments Co., Woburn, MA, can be used to microinject DNA into the pronuclei. DMEM, fetal bovine serum, and DPBS can be obtained from GIBCO Laboratories, Gaithersville, MD.

For construction of transgenic mice, procedures for embryo manipulation and microinjection are described in "Manipulating the Mouse Embryo" by B. Hogan, F. Costantini and E. Lacy (Cold Spring Harbor Laboratory, 1986). Similar methods are used for production of other transgenic animals. Mouse zygotes are collected from six week old females that have been superovulated with pregnant mares serum (PMS) followed 48 hours later with human chorionic gonadotropin. Primed females are placed with males and checked for vaginal plugs on the following morning. Pseudopr gnant females are selected for estrus, placed with proven st rile vasectomized males and used as

recipients. Zygotes are collected and cumulus cells removed by treatment with hyaluronidase (1 mg/ml). Pronuclear embryos are recovered from female mice mated to males. Females are treated with pregnant mare serum, PMS, (5 IU) to induce follicular growth and human chorionic gonadotropin, hCG (51 U) to induce ovulation. Embryos are recovered in a Dulbecco's modified phosphate buffered saline (DPBS) and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum.

Microinjections can be performed using Narishige micromanipulators attached to a Nikon diaphot microscope. Embryos are held in 100 microliter drops of DPBS under oil while being microinjected. DNA solution is microinjected into the largest visible male pronucleus. Successful injection is monitored by swelling of the pronucleus. Immediately after injection embryos are transferred to recipient females, mature mice mated to vasectomized male mice. Recipient females are anesthetized using 2,2,2-tribromoethanol. Paralumbar incisions are made to expose the oviducts and the embryos are transformed into the ampullary region of the oviducts. The body wall is sutured and the skin closed with wound clips. Recipients are appropriately ear notched for identification and maintained until parturition.

At three weeks of age about 2-3 cm long tail samples are excised for DNA analysis. The tail samples are digested by incubating overnight at 55°C in the presence of 0.7 ml 50 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS and 350 μg of proteinase K. The digested material is extracted once with equal volume of phenol and once with equal volume of phenol:chloroform (1:1 mixture). The supernatants are mixed with 70 μl 3 M sodium acetat (pH 6.0) and the DNAs are precipitated by adding equal volum of 100% ethanol. The DNAs are spun down in a microfuge,

washed once with 70% ethanol, dried and dissolv d in 100 μL TE buff r (10 mM Tris, pH 8.0 and 1 mM EDTA). 10 to 20 μl of DNAs were cut with *Bam*HI and *Bgl*11 or *Eco*RI, electrophoresed on 1% agarose gels, blotted onto nitrocellulose paper and hybridized with ^{32}P-labeled DNA sequences. Transgenic animals are identified by autoradiography.

The transgenic females are mated. At five days following parturition milk samples were taken and assayed for the fusion protein. At six to seven weeks of age transgenic males are mated. The F1 litters are analyzed for transgene. The positive females are kept and mated at five weeks of age. At five days following parturition milk samples are assayed for the fusion protein. Milk samples (50-200 μl) are collected from anesthetized mice injected with 0.05 units of oxytocin, an inducer of lactation. The milk is collected in a glass capillary with the aid of mammary palpation. The fusion protein is then isolated by binding to the HPC-4 antibody.

Purification using the HPC-4 antibody-epitope.

The antibody can be bound to a variety of substrates, for use in purification and isolation of the fusion protein, including agarose, acrylamide and other types of conventional chromatographic resins, filters, etc. These materials are known to those skilled in the art, as are the methods for attaching the protein to them. The selection of the material will depend in large part on the scale of the purification or the sample to be analyzed, as well as biocompatibility and government agency approval where the end-product is for pharmaceutical use.

Protease Cleavage Site.

In the most preferred embodiment, the fusion protein includes a protease cleavage site between the epitope and the protein to be isolated. Suitable sites include sequ nces cleaved by Factor Xa: Ile Glu

Gly Arg (IEGR), enterokinase: Asp Asp Asp Asp Lys (DDDDK), and thrombin: Phe/Gly Pro Arg (F/GPR).

Following purification with the HPC-4, the fusion protein is treated with the appropriate enzyme to cleave the binding peptide from the desired protein.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Construction of a vector for expression of a fusion truncated tissue factor.

Blood coagulation results from the production of thrombin, a proteolytic enzyme inducing platelet aggregation and cleaving fibrinogen to fibrin, which stabilizes the platelet plug. A number of proenzymes and procofactors circulating in the blood interact in this process through several stages during which they are sequentially or simultaneously converted to the activated form, ultimately resulting in the activation of prothrombin to thrombin by activated factor X (fXa) in the presence of factor Va, ionic calcium, and platelets.

Factor X can be activated by either of two pathways, termed the extrinsic and intrinsic pathways. The intrinsic pathway, or surface-mediated activation pathway, consists of a series of reactions where a protein precursor is cleaved to form an active protease, beginning with activation of factor XII to factor XIIa, which converts factor XI to factor XIa, which, in the presence of calcium, converts factor IX to factor IXa. Factor IX can also be activated via the extrinsic pathway by tissue factor (TF) in combination with activated factor VII (factor VIIa; fVIIa). The activated factor IX, in the presence of calcium, phospholipid (platelets), and factor VIIIa, activates factor X to factor Xa.

Physiologically, the maj r pathway involved in coagulation is believed to b the extrinsic pathway,

an essential step of which is activation of factor VII to factor VIIa. Clotting assays and other activity assays designed to measure factor VII and VIIa generally must employ TF, the cofactor required for factor VIIa coagulant activity. Most commonly, TF is provided as a relatively crude preparation known as thromboplastin. Tissue factor is an integral membrane glycoprotein having a protein and a phospholipid component. It has been isolated from a variety of tissues and species and reported to have a molecular mass of between 42,000 and 53,000. DNA encoding tissue factor and methods for expression of the protein have now been reported, for example, in European Patent Application 0 278 776 by Genentech, Inc. and by J. H. Morrissey, et al. Cell 50, 129-135 (1987).

The nucleotide (Sequence ID No. 2) and amino acid (Sequence ID No. 3) sequence of truncated tissue factor (tTF) is shown below, which, as described below, was modified from the sequence described in U.S. Serial No. 07/683,682 filed April 10, 1991, the teachings of which are incorporated herein. The truncated tissue factor protein lacks the predicted transmembrane and cytoplasmic domains of tissue factor. The essential difference between truncated tissue factor and wild-type tissue factor is that truncated tissue factor is no longer tethered to the phospholipid membrane surface. Soluble tissue factor is a cofactor for activated factor VII (FVII) but not precursor factor VII (FVII). Intact tissue faotor is a cofactor for FVII and FVIIa.

The following amino acid and nucleotide sequences encode a soluble form of truncated tissue factor. Nucleotide sequence is numbered sequentially on the left. Amino acid sequenc is given above the nucleotide sequence using th standard one-letter code, and is numbered on the right according to th

numbering of the mature tissue factor protein reported by Morrissey et al. Cell 50, 129-135 (1987). The *Sma*I site at the 5′ end, and the *Xba*I site at the 3′ end are underlined. The first three nucleotides of the *Sma*I site are removed upon digestion with the restriction enzyme, *Sma*I. This permits the tTF cDNA sequence to be blunt-end ligated to *Stu*I site of the pIN-III-*pelB*-HPC-4 expression vector in a manner that preserves the reading frame of the tTF cDNA clone.

```
                G  T  T  N  T  V  A  A  Y  N  L  T  W  K  S  T
1   CCCGGGACCACCAATACTGTGGCAGCATATAATTTAACTTGGAAATCAAC          +17
    SmaI

                N  F  K  T  I  L  E  W  E  P  K  P  V  N  Q  V
51  TAATTTCAAGACAATTTTGGAGTGGGAACCCAAACCCGTCAATCAAGTCT          +33

                Y  T  V  Q  I  S  T  K  S  G  D  W  K  S  K  C  F
101 ACACTGTTCAAATAAGCACTAAGTCAGGAGATTGGAAAAGCAAATGCTTT        +50

                Y  T  T  D  T  E  C  D  L  T  D  E  I  V  K  D  V
151 TACACAACAGACACAGAGTGTGACCTCACCGACGAGATTGTGAAGGATGT        +67

                K  Q  T  Y  L  A  R  V  F  S  Y  P  A  G  N  V
201 GAAGCAGACGTACTTGGCACGGGTCTTCTCCTACCCGGCAGGGAATGTGG        +83

                E  S  T  G  S  A  G  E  P  L  Y  E  N  S  P  E  F
251 AGAGCACCGGTTCTGCTGGGGAGCCTCTGTATGAGAACTCCCCAGAGTTC        +100

                T  P  Y  L  E  T  N  L  G  Q  P  T  I  Q  S  F  E
301 ACACCTTACCTGGAGACAAACCTCGGACAGCCAACAATTCAGAGTTTTGA        +117

                Q  V  G  T  K  V  N  V  T  V  E  D  E  R  T  L
351 ACAGGTGGGAACAAAAGTGAATGTGACCGTAGAAGATGAACGGACTTTAG        +133

                V  R  R  N  N  T  F  L  S  L  R  D  V  F  G  K  D
401 TCAGAAGGAACAACACTTTCCTAAGCCTCCGGGATGTTTTTGGCAAGGAC        +150

                L  I  Y  T  L  Y  Y  W  K  S  S  S  S  G  K  K  T
451 TTAATTTATACACTTTATTATTGGAAATCTTCAAGTTCAGGAAAGAAAAC        +167

                A  K  T  N  T  N  E  F  L  I  D  V  D  K  G  E
501 AGCCAAAACAAACACTAATGAGTTTTTGATTGATGTGGATAAAGGAGAAA        +183

                N  Y  C  F  S  V  Q  A  V  I  P  S  R  T  V  N  R
551 ACTACTGTTTCAGTGTTCAAGCAGTGATTCCCTCCCGAACAGTTAACCGG        +200
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                K  S  T  D  S  P  V  E  C  M  G  Q  E  K  G  E  F  +217
601  AAGAGTACAGACAGCCCGGTAGAGTGTATGGCCAGGAGAAAGGGGAATT

                R  E  stop                                      +219
651  TAGAGAATAACTGCAGTCTAGA
                    XbaI
```

Construction of pIN-III-*pelB* Expression Vector:

This vector is derived from pIN-III-ompA (John Ghrayeb et al, *EMBO J.* 3(10):2437-2442 (1984)). Cleavage of pIN-III-ompA with *Xba*I and *Bam*HI removes a DNA fragment which contains the Shine-Dalgarno sequence GAGG, and the entire nucleotide sequences encoding for the ompA signal peptide. As shown in Figure 1, by ligation of 8 overlapping oligonucleotides (four sense and the other four complementary antisense), a DNA fragment was synthesized which contains the missing Shine-Dalgarno sequence followed by oligonucleotides sequence encoding a 22 residue long peptide representing the *pelB* signal peptide (Sahu-Ping Lei, et al, J. Bacteriol. 169(9):4379-4383, 1987) (AA -22 to -1) (Sequence ID No. 4).

An *Eco*RI restriction site was included in this DNA fragment, immediately after the *pelB* leader sequence, which encodes two extra residues Glu, Phe (+1 and 2). The DNA fragment (Sequence ID No. 5) also encodes a 12 amino acid residue long peptide (residue 3 to 14), which is the epitope for the Ca^{++} dependent monoclonal antibody HPC-4. The *Eco*RI restriction site separates the HPC-4 epitope from the *pelB* signal peptide. Following the nucleotides encoding the epitope, are the nucleotides encoding the four amino acid residues Ile, Glu, Gly, and Arg, forming a factor Xa cleavage site (Sequence ID No. 6).

The factor Xa cleavage site is followed by a sequence containing several restriction enzyme sites for cloning of the target gene into this vector for expression. As shown in Figure 1, this DNA fragment contains an *Xba*I sticky end at the 5′ end and a *Bam*HI sticky end at the 3′ end for ligation to the *Xba*I and *Bam*HI sites of pIN-III-ompA. Multiple cloning sites of pIN-III-*pelB* includes *Stu*I, *Not* I, *Hind*III, and *Bam*HI. *Stu*I is a 6 bp blunt end cutter whose

recognition site is AGG CCT; when it cleaves this site it leaves three nucleotides at the 3′ end of the DNA fragment, AGG, which encode the last residue in the FXa cleavage site, Arg. Therefore, double digestion of this vector with *Stu*I and either one of the other 3′ end cloning sites (*Not*I or *Hin*dIII or *Bam*HI) provides a suitable directional cloning of the target gene for expression.

The oligonucleotide sequence of a synthetic DNA fragment encoding the *pelB* leader peptide (AA-22 to -1) (Sequence ID No. 4); HPC-4 epitope (AA +3 to 14) (Sequence ID No. 1); and the FXa cleavage site (AA 15 to 18) (Sequence ID No. 6), are shown below. The Shine-Dalgarno sequence GAGG (S-D) is overlined. The restriction enzyme sites useful for cloning have been outlined, which are *Eco*RI (between the *pelB* leader sequence and the HPC-4 epitope which creates two extra amino acids +1 and +2), *Stu*I, *Not*I, and *Hin*dIII. The *pelB* leader peptide cleavage site which is cleaved by bacterial signal peptidase is shown by an arrow. This DNA fragment was made by ligation of eight overlapping oligonucleotides so that it created a sticky *Xba*I site at the 5′ end and a sticky *Bam*HI site at the 3′ end for ligation into the *Xba*I and *Bam*HI site of the pIN-III-ompA vector. The boundary of oligonucleotides for the sense strand and at the bottom for the complementary antisense strand is shown by bold letters.

```
                                        -22
XbaI end          S-D          M
   5'-CTAGATAACGAGGGCAAAAAATGAAATACCTATTGCCTACGGCAGCCGC
       3'-TATTGCTCCCGTTTTTTACTTTATGGATAACGGATGCCGTCGGCG

                                                    -1 ↓+1
   TGGATTGTTATTACTCGCTGCCCAACCAGCCATGGCCGAATTCGAAGAT
   ACCTAACAATAATGAGCGACGGGTTGGTCGGTACCGGCTTAAGCTTCTA
                                          EcoRI

   CAGGTAGATCCGCGGTTAATCGATGGTAAGATTGAAGGAAGGCCTAGGC
   GTCCATCTAGGCGCCAATTAGCTACCATTCTAACTTCCTTCCGGATCCG
                                            StuI

   GGCCGCAAGCTTG-3'
   CCGGCGTTCGAACCTAG-5' BamHI end
   NotI   HindIII
```

Construction of pIN-III-*pelB*-tTF:

The construction of pIN-III-*pelB*-tTF is shown schematically in Figure 2. pJH27 is a plasmid that contains the entire truncated tissue factor (tTF) cDNA sequence cloned between *Bam*HI and *Xba*I restriction sites of the vector pGEM-7Zf(+) (Promega). Preparation of the tTF cDNA fragment with 5′ and 3′ ends compatible with cloning sites in the pIN-III-*pelB* expression vector was performed in a two-step process. At the first stage the plasmid pJH27 was digested with *Bbv*I restriction enzyme which cleaved away DNA sequences from the 5′ end of the cDNA coding for the tTF signal peptide and the first 11 residues of the mature tTF protein. In order to repair the tTF gene two complementary oligonucleotides were synthesized, which coded for the 11 missing residues and created a blunt, half-*Sma*I site at the 5′ end. This oligonucleotide contained a sticky end that could hybridize to, and therefore be ligated to, the sticky end of the tTF cDNA sequence created by *Bbv*I digestion.

In the second stage, the modified cDNA insert was removed from pJH27 by digestion with *Xba*I, and the

insert was resolved by agarose gel el ctrophoresis and purified by elution from the agarose gel. The resulting cDNA fragment which encodes the entire tTF was subcloned into the *Sma*I and *Xba*I sites of pUC19 plasmid. As shown in Figure 2, the tTF cDNA fragment then was removed from pUC19 with *Sma*I and *Hin*dIII restriction enzymes and subcloned into the *Stu*I and *Hin*dIII sites of the pIN-III-*pelB* expression vector.

Example 2: Expression and Isolation of the Fusion Protein.

Growth of Bacteria for Periplasmic Extract: The XL1-B strain of *E. coli* was grown at 37°C in LB media. 500 µl of overnight culture was transferred to a 125 ml flask containing 25 ml of LB and 100 µg/ml ampicillin. The flask was shaken at 37°C for 2-3 hrs until the OD_{600} is equal to 0.6 to 0.7. One liter LB media with ampicillin (Amp) was inoculated with the entire 25 ml bacterial culture and the shaking was continued at 37°C until the OD_{600} = 0.6 to 0.7. The culture then was induced with 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and the shaking was continued for another 8 hrs at room temperature. The bacterial culture was centrifuged at approximately 3000 rpm for 20 min to separate the culture medium from the cells. The cell pellet was resuspended with 50 ml of cold water and incubated for 1/2 hour on ice with shaking. The periplasmic extract was then collected by spinning at 10,000 g for 1/2 hr.

Approximately three-fourths of the protein was expressed in the media of the *E. coli*, and one-fourth was recovered from the periplasmic space after hypotonic shock. The periplasmic extract was mixed with the culture medium (which has been clarified by centrifugation, as described above), and the mixture was concentrated to approximately one-tenth the original volume using an ultrafiltrati n spiral cartridge concentrator with a 3000 MW cut-off membrane

(Amicon). Following concentration, this material was brought up to 0.1 M NaCl, 0.02 M Tris and 1 mM calcium chloride, and loaded on a HPC-4 column equilibrated with the same buffer (5 mg/ml HPC-4 IgG immobilized on Affi-Gel™ 10; 4 ml total used for 1 liter of starting bacterial culture), washed with approximately 200 ml 1 M NaCl, 0.02 M Tris-HCl, pH 7.5 containing 1 mM $CaCl_2$, followed by approximately 10 to 20 ml of the same buffer but with 0.1 M NaCl, and the protein eluted with 0.1 M NaCl, 0.02 M Tris-HCl, 5 mM EDTA, pH 7.5. Elution of the tTF from the column was monitored by absorbance of light at 280 nm; a single peak of protein was observed.

SDS-PAGE:

SDS-PAGE analysis (10% acrylamide using Laemmli system, Laemmli, Nature 227:680-685 (1970)) of the peak fraction from the affinity chromatography indicated a single monomeric band at around 30 kd in both reducing and non-reducing conditions, as shown in Figure 3. This is consistent with the expected molecular weight of tTF when it is not glycosylated.

Approximately 1 mg of soluble tissue factor is recovered from 1 liter of starting bacterial culture, and is homogeneous as shown by SDS gel electrophoresis. The tissue factor runs as monomer in the gel even without disulfide bond reduction. The soluble, truncated tissue factor protein isolated in this manner has full cofactor activity toward factor VIIa even without the HPC-4 epitope removed; this activity is equivalent to that of truncated tissue factor without the HPC-4 epitope expressed in mammalian cells, as shown by Figure 4.

Functionally the protein is equivalent to the protein expressed in mammalian c ll culture, but at less than 10% the cost. This functional equivalence is demonstrat d by the factor VIIa concentration dep nd nce on factor X activation and by the ability

of the tissue factor from both sources to increase factor VIIa amidolytic activity equivalently.

Example 3: Isolation of other proteins using the fusion protein technology in combination with the HPC-4 epitope.

Thrombomodulin fragments, EGF domains of factor X and EGF domains of protein C with the epitope linked to the amino terminal region of these proteins have been expressed in and recovered from other *E. coli* periplasmic space expression systems. A fragment of the thrombomodulin of the 4th-6th EGF domains has also been isolated from cultured mammalian cells in a single step purification on the antibody column as described above. In this case, the DNA encoding the thrombomodulin 4th-6th EGF domains was amplified from cDNA by PCR and ligated to synthetic oligonucleotides coding for the transferrin signal peptide followed by the HPC-4 epitope and Xa cleavage site. The resulting DNA fragment was subcloned into a pRc/RSV mammalian cell expression vector for expression in human 293 cells.

Modifications and variations of the method and compositions described herein will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Oklahoma Medical Research Foundation

(ii) TITLE OF INVENTION: Expression and Purification of Recombinant Soluble Tissue Factor

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kilpatrick & Cody
(B) STREET: 1100 Peachtree Street, Suite 2800
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: U.S.
(F) ZIP: 30309-4530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: OMRF130

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404-815-6508
(B) TELEFAX: 404-815-6555

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mouse

(ix) FEATURE:
(A) NAME/KEY: Binding-site
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Epitope recognized by HPC4 antiprotein C antibody"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 672 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1..6

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 67..72

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AAGTCAGGAG ATTGGAAAAG CAAATGCTTT TACACAACAG ACACAGAGTG TGACCTCACC 180

GACGAGATTG TGAAGGATGT GAAGCAGACG TACTTGGCAC GGGTCTTCTC CTACCCGGCA 240

GGGAATGTGG AGAGCACCGG TTCTGCTGGG GAGCCTCTGT ATGAGAACTC CCCAGAGTTC 300

ACACCTTACC TGGAGACAAA CCTCGGACAG CCAACAATTC AGAGTTTTGA ACAGGTGGGA 360

ACAAAAGTGA ATGTGACCGT AGAAGATGAA CGGACTTTAG TCAGAAGGAA CAACACTTTC 420

CTAAGCCTCC GGGATGTTTT TGGCAAGGAC TTAATTTATA CACTTTATTA TTGGAAATCT 480

TCAAGTTCAG GAAAGAAAAC AGCCAAAACA AACACTAATG AGTTTTTGAT TGATGTGGAT 540

AAAGGAGAAA ACTACTGTTT CAGTGTTCAA GCAGTGATTC CCTCCCGAAC AGTTAACCGG 600

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CTGCAGTCTA GA 672

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 218 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln Val
            20                  25                  30

Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys Cys
        35                  40                  45

Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val Lys
    50                  55                  60

Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala Gly
65                  70                  75                  80

Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn Ser
                85                  90                  95

Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr Ile
            100                 105                 110

Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu Asp
        115                 120                 125

Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg Asp
    130                 135                 140

Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser Ser
145                 150                 155                 160

Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu Ile
                165                 170                 175

Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val Ile
            180                 185                 190
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Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu Cys
195 200 205

Met Gly Gln Glu Lys Gly Glu Phe Arg Glu
210 215

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 10..13
(D) OTHER INFORMATION: /note= "Shine-Dalgarno sequence"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 22..85
(D) OTHER INFORMATION: /note= "pelB leader peptide"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..14
(D) OTHER INFORMATION: /note= "HPC-4 epitope"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 15..18
(D) OTHER INFORMATION: /note= "Factor Xa cleavage site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 146..152
(D) OTHER INFORMATION: /note= "NotI restriction enzyme"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 83..88
(D) OTHER INFORMATION: /note= "EcoRI restriction enzyme site"

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(B) LOCATION: 134..139
(D) OTHER INFORMATION: /note= "StuI restriction enzyme site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 150..155
(D) OTHER INFORMATION: /note= "HindIII restriction enzyme"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Cleavage-site
(B) LOCATION: 1..4
(D) OTHER INFORMATION: /note= "Factor Xa Cleavage Site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Cleavage-site
(B) LOCATION: 1..5
(D) OTHER INFORMATION: /note= "Enterokinase Cleavage Site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Cleavage-site
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /note= "Thrombin Cleavage Site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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1

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Cleavage-site
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /note= "Thrombin Cleavage Site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Pro Arg
1

We claim:

1. A fusion protein comprising an epitope bound in the presence of calcium by the monoclonal antibody designated as HPC-4, deposited with the American Type Culture Collection, Rockville, MD, on November 2, 1988, and assigned ATCC No. HB 9892, and a protein to be isolated by binding of the fusion protein to HPC-4 antibody.

2. The fusion protein of claim 1 wherein the epitope consists of the twelve amino acid sequence E D Q V D P R L I D G K.

3. The fusion protein of claim 1 further comprising a specific protease cleavage site between the epitope and the protein to be isolated.

4. The fusion protein of claim 3 wherein the protease cleavage site is selected from the group of amino acid sequences specifically cleaved by Factor Xa: Ile Glu Gly Arg (IEGR), enterokinase: Asp Asp Asp Asp Lys (DDDDK), and thrombin: Phe/Gly Pro Arg (F/GPR).

5. The fusion protein of claim 1 wherein the protein to be isolated is selected from the group consisting tissue factor and thrombomodulin 4-6.

6. The fusion protein of claim 1 consisting essentially of the twelve amino acid sequence E D Q V D P R L I D G K at the N-terminus, an amino acid sequence specifically cleaved by factor Xa, and tissue factor.

7. The fusion protein of claim 6 wherein the tissue factor is soluble tissue factor.

8. An isolated nucleic acid sequence encoding a fusion protein comprising an epitope bound in the presence of calcium by the monoclonal antibody designated as HPC-4, deposited with the American Type Culture Collection, Rockville, MD, on November 2, 1988, and assigned ATCC No. HB 9892, and a protein to be isolated by binding of the fusion protein t HPC-4 antibody.

9. The nucleic acid sequenc of claim 8 wherein the epitope consists of the twelv amino acid sequence E D Q V D P R L I D G K.

10. The nucleic acid sequence of claim 8 further comprising a specific protease cleavage site between the epitope and the protein to be isolated.

11. The nucleic acid sequence of claim 8 wherein the protease cleavage site is selected from the group of amino acid sequences specifically cleaved by Factor Xa: Ile Glu Gly Arg (IEGR), enterokinase: Asp Asp Asp Asp Lys (DDDDK), and thrombin: Phe/Gly Pro Arg (F/GPR).

12. The nucleic acid sequence of claim 8 wherein the protein to be isolated is selected from the group consisting tissue factor and thrombomodulin EGF domains 4-6.

13. The nucleic acid sequence of claim 8 consisting essentially of the twelve amino acid sequence E D Q V D P R L I D G K at the N-terminus, an amino acid sequence specifically cleaved by factor Xa, and tissue factor.

14. The nucleic acid sequence of claim 13 wherein the tissue factor is soluble tissue factor.

15. The nucleic acid sequence of claim 13 inserted into a vector for expression in cells selected from the group consisting of procaryotic, yeast, and mammalian cells.

16. A method for making a protein that can be purified by affinity binding to the monoclonal antibody designated as HPC-4, deposited with the American Type Culture Collection, Rockville, MD, on November 2, 1988, and assigned ATCC No. HB 9892 in the presence of calcium comprising expressing a fusion pr tein comprising an epitope bound in the presence of calcium by HPC-4, and a protein to b isolated, from a sequence encoding the protein, inserted into a vector in an expressi n system.

17. The method of claim 16 wherein the expression system is selected from the group consisting of eukaryotic cells, yeast cells, and mammalian cells in culture.

18. The method of claim 16 further comprising expressing a selective protease cleavage site in the fusion protein between the epitope and the protein to be isolated.

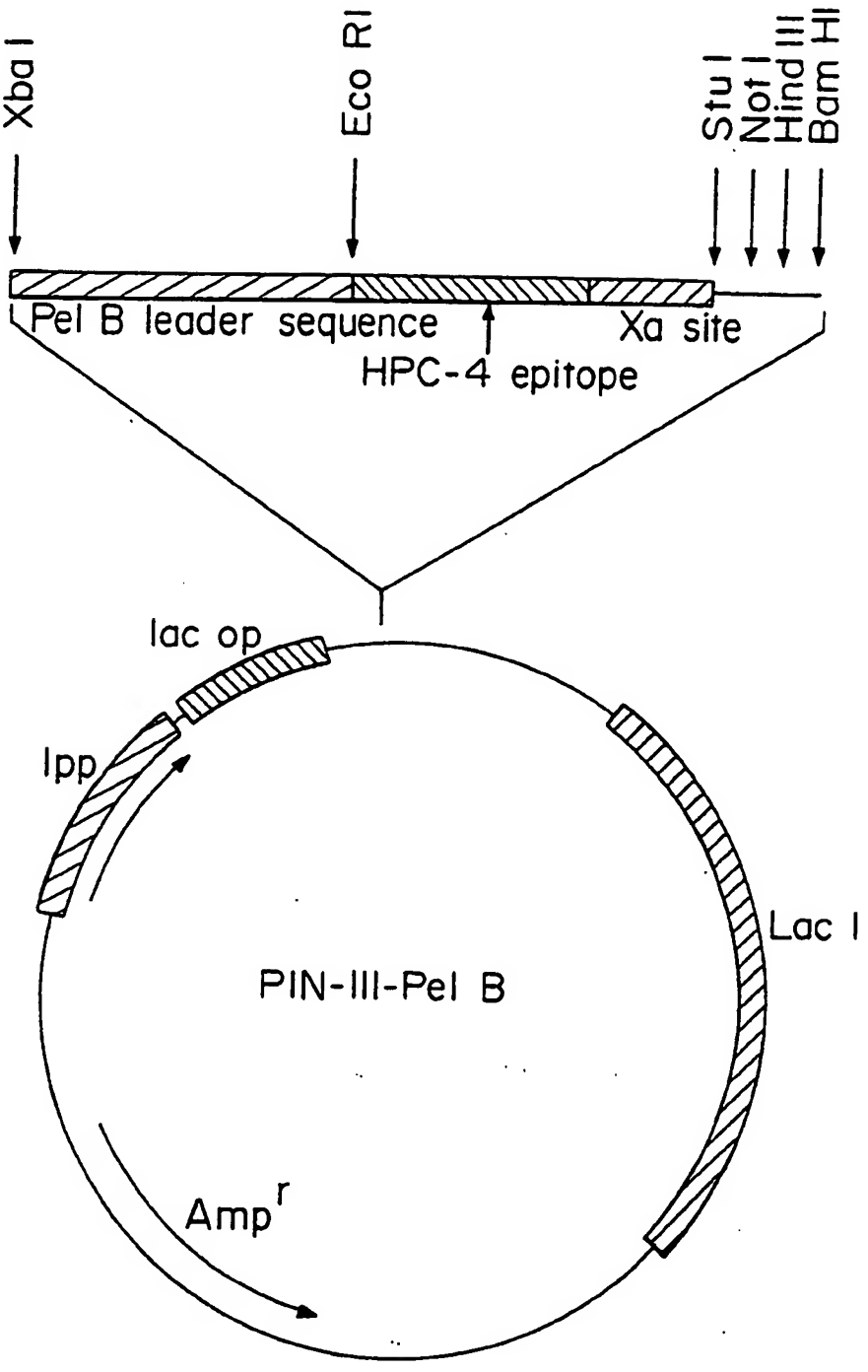
19. The method of claim 16 further comprising isolating the fusion protein by binding the protein to immobilized HPC-4 antibody in the presence of calcium, removing unbound protein by washing the immobilized HPC-4 antibody with a calcium containing solution, and eluting the fusion protein from the immobilized HPC-4 antibody by washing the immobilized HPC-4 antibody with a solution removing calcium from the bound fusion protein.

20. The method of claim 19 further comprising removing the HPC-4 epitope from the fusion protein.

21. The method of claim 20 wherein the fusion protein includes a specific protease cleavage site between the epitope and the protein to be isolated, further comprising cleaving the epitope from the protein to be isolated using the protease either free or immobilized.

22. The method of claim 16 wherein the protein to be isolated is selected from the group consisting tissue factor and thrombomodulin.

23. The method of claim 21 wherein the protease is selected from the group consisting of factor Xa, enterokinase and thrombin.


Xba I
Eco RI
Stu I
Not I
Hind III
Bam HI
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Xa site
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lpp
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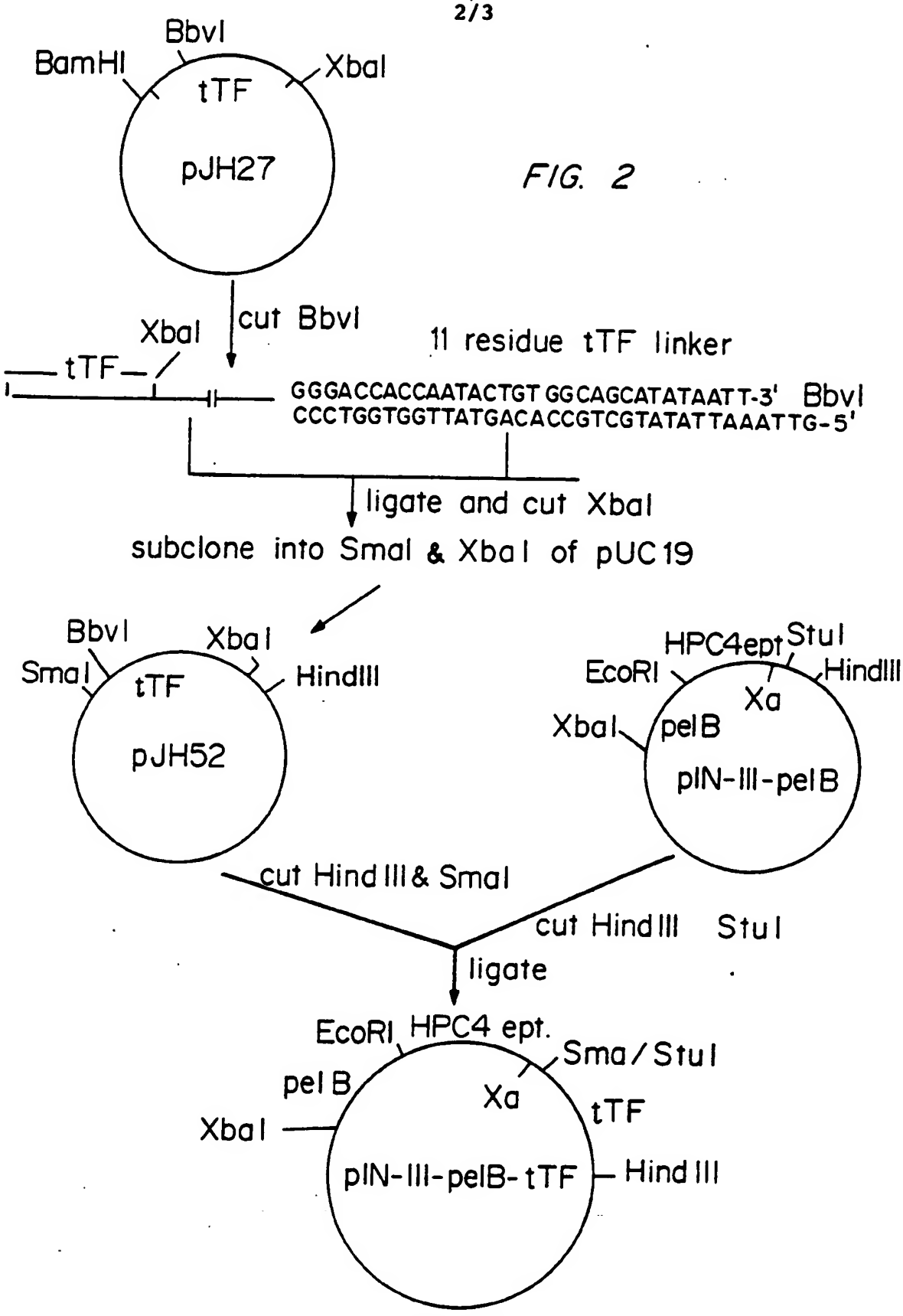
Amp r

FIG. 1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tTF
pJH27
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tTF
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CCCTGGTGGTTATGACACCGTCGTATATTAAATTG-5'
ligate and cut XbaI
subclone into SmaI & XbaI of pUC19
BbvI
XbaI
SmaI
tTF
HindIII
pJH52
HPC4ept
StuI
EcoRI
HindIII
Xa
XbaI
pelB
pIN-III-pelB
cut HindIII & SmaI
cut HindIII StuI
ligate
EcoRI
HPC4 ept.
Sma/StuI
pelB
Xa
tTF
XbaI
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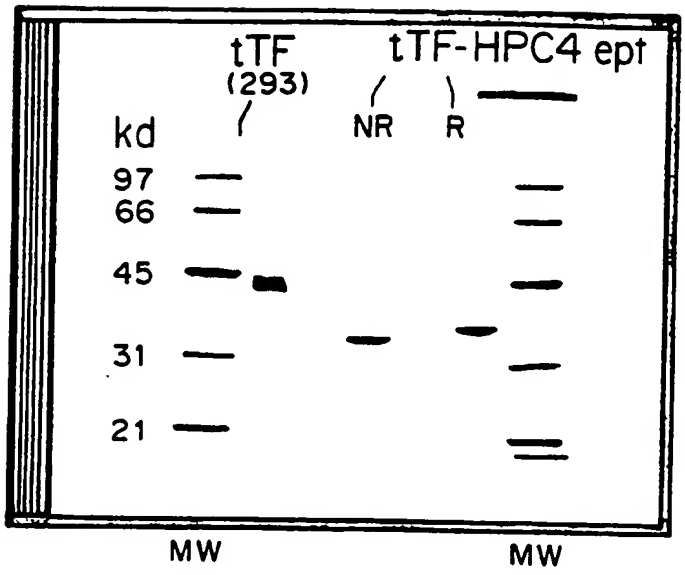
HindIII

FIG. 2

FIG. 3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kd
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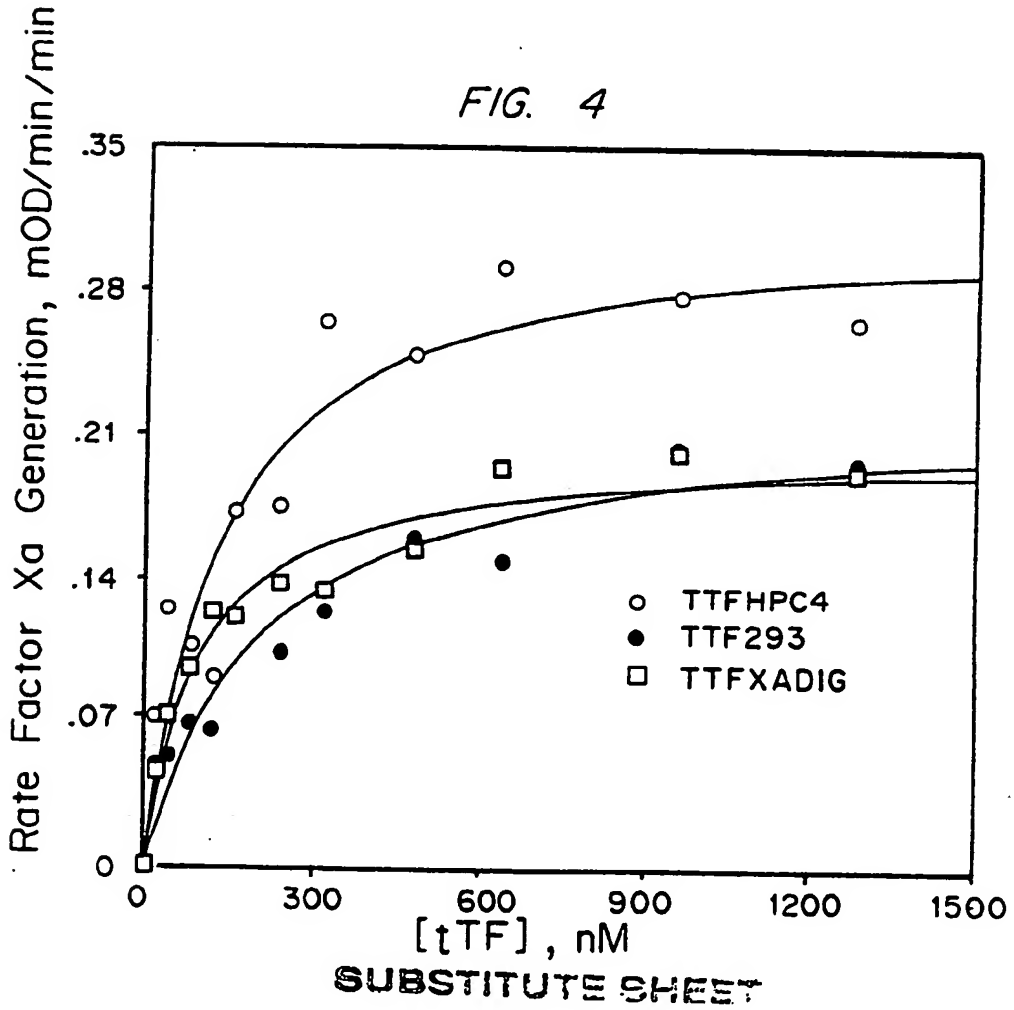
MW
MW

FIG. 4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TTF293
TTFXADIG

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/11270

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 15/62, C07K 3/20, C07K 15/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A1, 9007524 (OKLAHOMA MEDICAL RESEARCH FOUNDATION), 12 July 1990 (12.07.90), abstract --	1-23
Y	WO, A1, 8804692 (IMMUNEX CORPORATION), 30 June 1988 (30.06.88), claims 1,5,6,7, abstract -- --------	1-23

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1993	27 APR 1993
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Auth rized officer MIKAEL G:SON BERGSTRAND

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/02/93

International application No.
PCT/US 92/11270

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9007524	12/07/90	AU-A-	4968390	01/08/90
		CA-A-	2006684	30/06/90
		EP-A-	0407544	16/01/91
		JP-T-	3504332	26/09/91
		US-A-	5147638	15/09/92
WO-A1- 8804692	30/06/88	AU-A-	1056188	15/07/88
		EP-A-	0335899	11/10/89
		JP-T-	2501112	19/04/90
		US-A-	4851341	25/07/89
		US-A-	5011912	30/04/91